Patent
Attorney's Docket No. 0070777-000014

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Ju-Ock NAM et al.	)	Group Art Unit: 1654
	)	
Application No.: 10/552,291	)	Examiner: Christina Bradley
	)	
Filed: October 3, 2005	)	Confirmation No.: 6194
	)	
For: USE OF A PEPTIDE THAT INTERACTS WITH ALPHA V BETA 3 INTEGRIN OF ENDOTHELIAL CELL	)	

DECLARATION OF DR. In-San Kim

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Dr. In-San Kim, declare the following:

2. I am a citizen of the Republic of Korea, and have the following mailing address: Cell and Matrix Research Institute; Department of Biochemistry and Cell Biology, Kyungpook National University School of Medicine, 101 Dongin-Dong, Jung-gu, Daegu, 700-422, Republic of Korea;

3. I graduated from Kyungpook National University School of Medicine with M.D. and a Ph.D. degrees in 1989;

4. I am a professor in the Department of Biochemistry and cell Biology of Kyungpook National University School of Medicine;

5. I have read and am familiar with the above-identified United States patent application filed October 3, 2004, and I am submitting this Declaration in support of that application;

6. I have performed and/or supervised the experiments reported below:

In order to examine whether the fas-1 domain comprising peptide of the present invention (YH18) has anti-tumor activity, the inventors of the above-identified application were performed the following example.

Example : Analysis of anticancer effect of fas-1 domain

1-1: Analysis of proliferation of fas-1-overexpressing melanoma cells

In order to examine whether the fas-1 domain comprising peptide of the present invention (YH18) is involved in the proliferation of melanoma cells, melanoma cells that overexpress the fas-1 domain were prepared and a test was performed using the cells.

cDNA encoding the fourth fas-1 domain of βig-h3 was cloned into the *Eco*RI/*Xho*I sites of a pLNCX retrovirus vector (Clontech Lab. Inc., USA). The amino acid sequence of fourth fas-1 domain of βig-h3 and cDNA sequence encoding the amino acid sequence of fourth fas-1 domain are as follow:

fourth fas_1 domain comprising peptide of the present invention (SEQ ID NO.: 6 of the present invention)

MGTVMDVLKGDNRFSMLVAAIQSAGLTETLNREGVYTVFAPTNEAFRALPPRERSRLLGDA
KELANILKYHIGDEILVS**KELANILKYHIGDEILVS**GGIGALVRLKSLQGDKLEVSLKNNVVSV
NKEPVAEPDIMATNGVVHVITNVL

cDNA sequence encoding the fourth fas-1 domain

atggggactg tcatggatgt cctgaaggga gacaatcgct ttagcatgct ggtagctgcc

atccagtctg caggactgac ggagaccctc aaccgggaag gagtctacac agtctttgct

cccacaaatg aagccttccg agccctgcca ccaagagaac ggagcagact c**ttgggagat**

gccaaggaac ttgccaacat cctgaaatac cacattggtg atgaaatcct ggttagcgga

ggcatcgggg ccctggtgcg gctaaagtct ctccaaggtg acaagctgga agtcagcttg

aaaaacaatg tggtgagtgt caacaaggag cctgttgccg agcctgac atcatggcca

caaatggcgt ggtccatgtc atcaccaatg ttctg

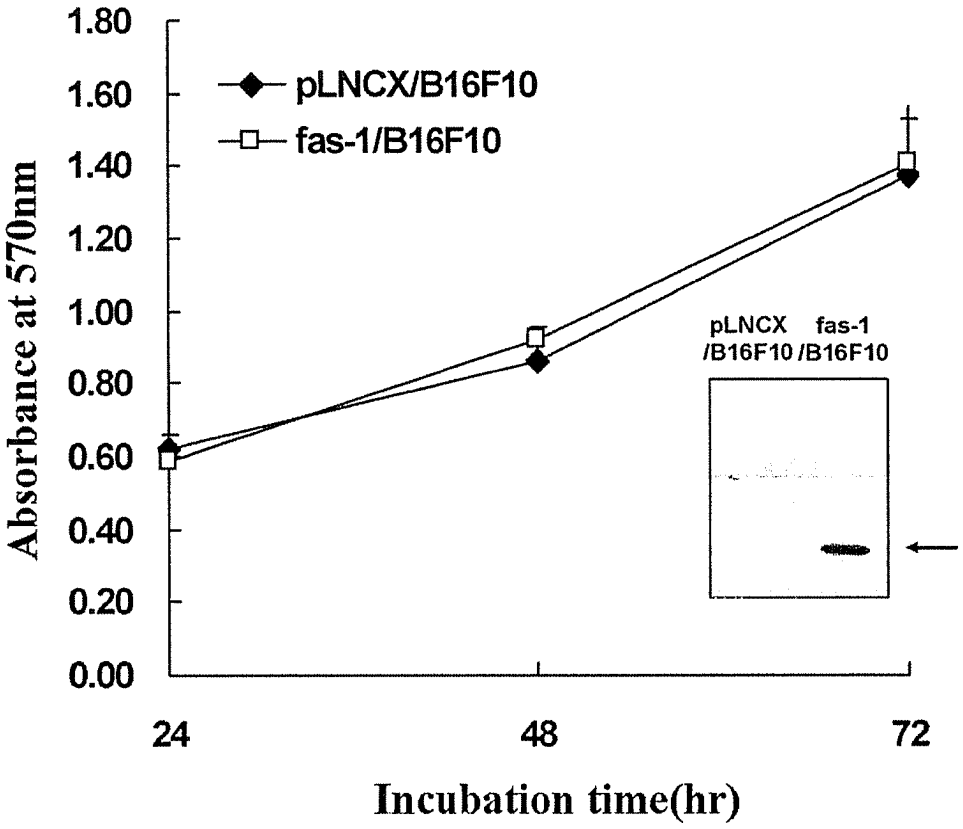
The shaded and underlined peptide and cDNA sequences are included in the sequence listing of the invention.

The resulting recombinant retrovirus vector was named "fas-1/pLNCX". Packaging cell PT67 (Clontech Lab. Inc., USA) were transfected with the fas-1/pLNCX vector using lipofectamine. The transformed cells were incubated at 37 °C for 48 hours. After completion of the incubation, virus particles present in the culture medium were collected, and filtered one time through a 0.45-μm filter. The filtered virus particles were added to a culture medium of melanoma cells (B16F10), and then allowed to react at 37°C for 6 hours. The culture medium was replaced with fresh medium, and the transfected cells were screened several times in the presence of 1 mg/ml of G418. Western blot analysis using a βig-h3 antibody was performed to confirm that the fas-1 domain is overexpressed in the screened transfected cells. As a result, fas-1-overexpressing melanoma cells (hereinafter, referred to as "fas-1/B16F10") were prepared. As a control, melanoma cells (pLNCX/B16F10) that overexpress an empty vector (pLNCX) containing no fas-1 cDNA were used.

Each of the fas-1-overexpressing melanoma cells (fas-1/B16F10) and the control cells (pLNCX/B16F10) was suspended in medium at a density of 3×10^4 cells/ml, and 0.1 ml of the cell suspension was added to each well of a 96-well plate. The cells were incubated at 37 °C for 48-72 hours. 50 μl of 0.5 mg/ml thiazolyl blue (MTT; Sigma) solution was added to each well and allowed to react at 37 °C for 4 hours. After completion of the reaction, the medium was removed, and each well was added with DMSO and left to stand at room temperature for a few minutes. Thereafter, the absorbance was measured at 570 nm in a Bio-Rad model 550 microplate reader.

The results showed that, as shown in FIG. 1a, there was no difference in the proliferation between the fas-1/B16F10 cells and the pLNCX/B16F10 cells. This confirms that the fas-1 domain has no effect on the proliferation of melanoma cells.

<Fig. 1a>



1-2: Analysis of growth of tumors derived from fas-1-overexpressing melanoma cells

In present invention, whether the fas-1 domain influences the growth of tumors derived from the fas-1-overexpressing melanoma cells prepared in Example 1-1 above was tested *in vivo*.

Each of the fas-1-overexpressing melanoma cells (fas-1/B16F10) and the control cells (pLNCX/B16F10) was suspended in medium at a density of 1×10^7 cells/ml. 0.1 ml of each of the cell suspensions was injected subcutaneously to 5-6 week old mice (Hyochang scientific company, Korea) to make a tumor model. Since the diameter of the tumor reached about 3 mm, the body weight and tumor size of the mice were checked at intervals of three days. The longest diameter and the shortest diameter of the tumor were measured with slide calipers, and the measured values were substituted in the following equation (1) to calculate the volume of the

tumor.

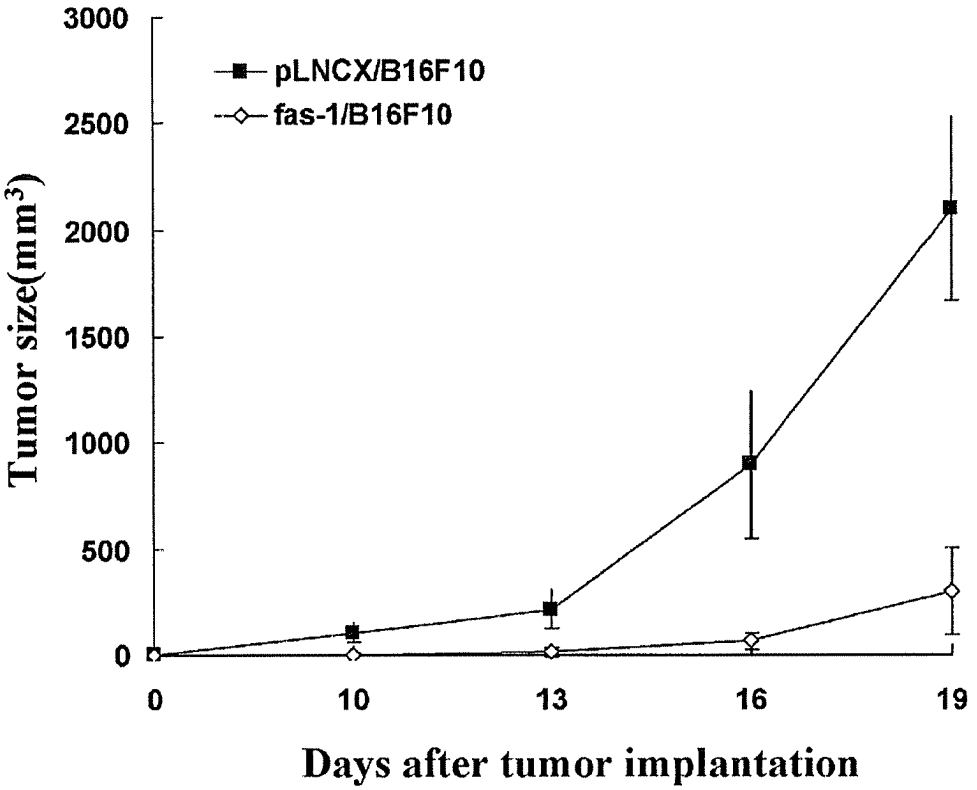
[Equation 1]

Volume of tumor = $a \times b^2/2$

Wherein, 'a' is the shortest diameter of the tumor, and 'b' is the longest diameter of the tumor.

The results showed that, as shown in FIG. 1b, the size of the tumor derived from the pLNCX/B16F10 cells was increased rapidly with passage of time from 13 days after production of the tumor model. On the other hand, the tumor derived from the fas-1/B16F10 cells showed little or no increase in size even at 13 days after production of the tumor model, and then, the increase of tumor growth with the passage of time was not observed.

<Fig. 1b>



Such results confirm that the fas-1 domain does not affect the *in vitro* proliferation of melanoma cells, but it inhibits the *in vivo* growth of tumors derived from the melanoma cells.

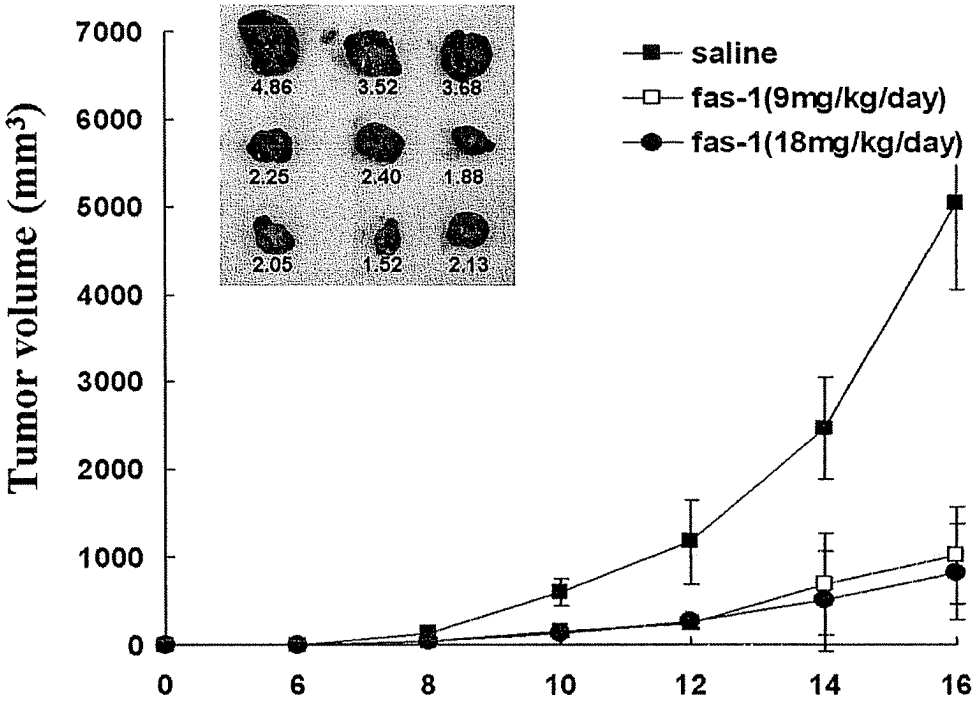
1-3: Test of tumor growth inhibitory effect of fas-1 domain

To further confirm the inhibitory effect of the fas-1 domain against tumor growth, the present inventors performed a test using BALB/c nude mice.

Melanoma cells B16F10 were suspended in medium at a density of 1 x 10^7 cells/ml. 0.1 ml of the cell suspension was injected subcutaneously to 5-6 week old BALB/c nude mouse (purchased from Jung-Ang Animal Inc. Ltd., Korea) to make a subcutaneous tumor model. When the volume of the tumor reached about 25 mm^3, mice having similar tumor size were selected and divided into three groups. The fas-1 domain (βig-h3 D-IV) was injected into the abdominal cavity of the mice of each group at the amount of 9 mg/kg or 18 mg/kg one time a day. A control was injected with saline solution. Thereafter, the body weight and tumor size of the mice were checked at intervals of three days. The diameter of the tumor was measured with slide calipers, and the volume of the tumor was calculated according to the equation 1 above.

The results showed that, as shown in FIG. 1c, the tumor in the control group started to grow rapidly from 10 days after induction of the tumor from the melanoma cells. On the other hand, the tumor in the test group injected with the fas-1 domain showed little or no increase in size even at 10 days after tumor induction, and then, its growth rate with time was much lower than that of the control.

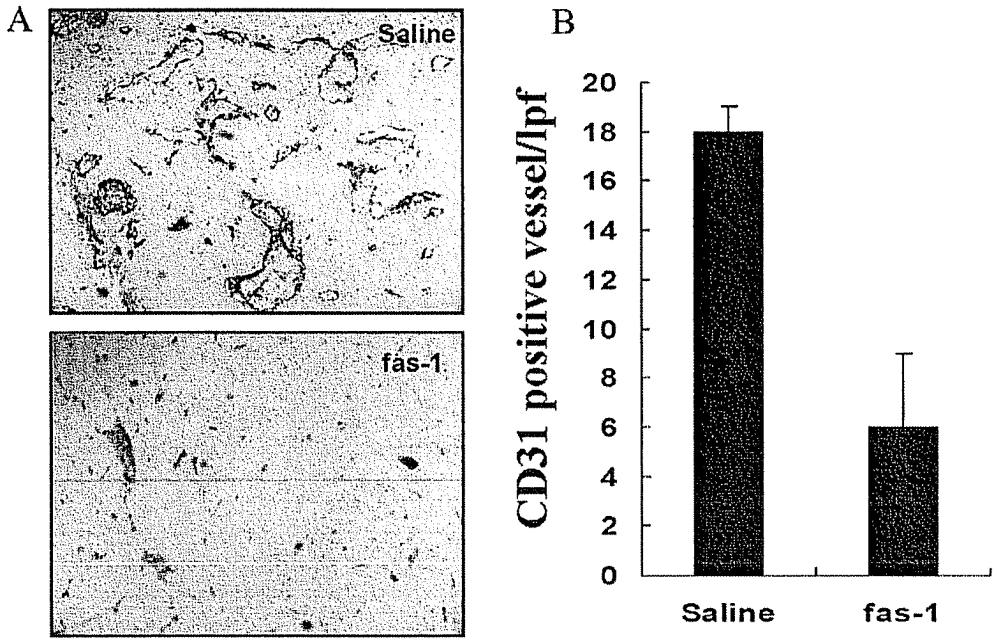
<Fig. 1c>



Meanwhile, the tumor was removed after a given time, photographed with a stereoscopic microscope, weighed, and then fixed in 4% paraformaldehyde. The fixed tumor was immersed in 30%, 20% and 10% sucrose solutions one after another for one hour each solution, frozen rapidly with liquid nitrogen spray, and then left to stand at 80 °C for two hours. The frozen tumor was sliced with a tissue microtome (Leica, Germany) to make tissue slides which were then dried at room temperature. Thereafter, the tissue slides were immuno-stained with CD31 (Pharmingen, San Diego, CA), an antibody that binds specifically to vascular endothelial cells. The immuno-stained slides were observed under a microscope, and the number of blood vessels from 3-5 LPF (low power fields; x100) was counted and averaged. Each group consisted of 5 or 6 members.

The results showed that, as shown in FIG. 1d, the number of blood vessels stained with the antibody in the test group treated with the fas-1 domain was significantly smaller than that of the control. This suggests that fas-1 domain inhibits the growth of tumors by inhibiting angiogenesis.

<Fig. 1d>



7. The results detailed above demonstrate that a peptide according to the present invention can be used to treat cancer in a well-accepted arthritis animal model;

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: June 24 2008 By: _____
In San Kim